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Case report

An UPLC–MS/MS method for the determination of valproic acid in blood of a fatal intoxication case

Paula Proença Biochem. D [a,*], João Miguel Franco MSc [a], Carla Mustra MSc [a], Mariana Marcos Biochem. D [a], Ana Rita Pereira MD [a], Francisco Corte-Real MD, PhD, Professor, Director [a,b], Duarte Nuno Vieira MD, PhD, Professor, President [a,b]

[a] National Institute of Legal Medicine – Centre Branch, Largo da Sé Nova, 3000-213 Coimbra, Portugal
[b] Faculty of Medicine, University of Coimbra, Largo da Sé Nova, 3000-213 Coimbra, Portugal

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ABSTRACT

Valproic acid (VPA) has been used as an anticonvulsant for the treatment of epilepsy. The authors present a fatal case involving a 45-year-old female, found dead lying in bed with empty tablets of *Diplexil*® next to her. She was a chronic alcoholic and epileptic who had been under psychiatric treatment, having repeatedly demonstrated intent to commit suicide.

A rapid method was developed and validated to determine VPA in blood by ultra-performance liquid chromatography (UPLC) coupled with tandem mass spectrometry (MS/MS) with electrospray ionization source in negative ion mode.

The method involved sample treatment with phosphoric acid followed by solid-phase extraction. Chromatographic separation was achieved using an Acquity UPLC® BEH (2.1 × 50 mm id, 1.7 μm) column and a mobile phase containing ammonium acetate and acetonitrile, at a 0.5 mL/min flow rate. Detection and quantification of VPA was achieved using multiple reaction monitoring (MRM). The MS/MS transitions used for monitoring were *m/z* 143.1–143.1 for valproic acid and *m/z* 296.1–205.0 for hydrochlorothiazide used as an internal standard (IS).

The limit of quantification (LOQ) was 0.5 μg/mL and the method was linear in the concentration range of 0.5–100 μg/mL. The coefficients of variation obtained for accuracy and precision were less than 10% and the mean recovery was 95% for the three concentrations levels studied (5 μg/mL, 10 μg/mL and 50 μg/mL). Toxicological results showed high concentration of VPA (556 μg/mL) and therapeutic concentrations of tiapride, mirtazapine, oxazepam and nordiazepam. Blood sample analysis also revealed the presence of ethanol at a concentration of 1.34 g/L.

A specific, selective and sensitive method for the determination of VPA in blood was developed and can be used in routine forensic investigation. Toxicological results led the pathologist to rule that death was due to an intoxication caused by the simultaneous ingestion of high VPA concentrations and alcohol, with a suicidal legal-medical etiology.

1. Introduction

VPA (2-propylpentanoic acid), a carboxylic acid derivative synthesized in 1881, has been used since 1967 as an anticonvulsant for the treatment of epilepsy.[1] More recently, it has been employed in the treatment of bipolar affective disorder and other psychiatric diseases.[2–5] Furthermore, epilepsy often coexists with psychiatric illness, including depression.[6,7] Trade names for VPA products in Portugal are *Diplexil*® (as sodium salt) and *Depakine*® (as a complex of VPA and sodium valproate). Sodium valproate is the sodium salt of VPA which dissociates to the valproate ion in the gastrointestinal tract and exists as valproate ion in blood.

VPA pharmacological effects involve a variety of mechanisms, including increased gamma-aminobutyric acid (GABA)-ergic transmission, reduced release and/or effects of excitatory amino acids, blockade of voltage-gated sodium channels and modulation of dopaminergic and serotoninergic transmission.[8] The oral bioavailability of VPA approaches 100%. VPA is a weak acid, with a pk_a 4.8 and is highly bound to plasma proteins with an apparent volume of distribution of 0.1–0.5 L/kg. VPA is extensively metabolized by

* Corresponding author. Tel.: +351 239854230; fax: +351 239820549.
E-mail address: paulaproenca@dcinml.mj.pt (P. Proença).

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microsomal glucuronide conjugation, mitochondrial beta-oxidation and cytochrome P450-dependent omega-, (omega-1)- and (omega-2)-oxidation. After therapeutic dosing, VPA is rapidly absorbed from the gastrointestinal tract, with peak serum levels occurring 1–4 h after ingestion. VPA is metabolized extensively by the liver. The majority of VPA (70–80%) is eliminated in urine as metabolites within 24 h, but only 1–4% of dose is excreted unchanged.[1,9–12] The major urinary metabolite is the glucuronide conjugate of the parent drug, accounting for up to 59% of a dose. Other metabolites include 3-ketovalproic acid (23%), 3-, 4- and 5-hydroxyvalproic acid (3% each) and 2-propylglutaric acid (PGA) (5%); each of these, as well as the parent drug, may be excreted in conjugated form[1,13] (Fig. 1).

Several methods have been published for the determination of VPA in biological matrices. Liquid chromatography (LC), with UV or fluorescence detection[14–19] offer sufficient sensitivity but require prior derivatization of the drug to add a suitable chromophore or fluorophore. Amini et al.[18] have determined VPA by LC without prior derivatization. The desired sensitivity has been achieved using gas chromatography with mass spectrometry (GC–MS)[20–27] however, prior derivatization limits its suitability for routine sample analysis. More recently, LC–MS and LC–MS/MS methods[28–34] have been developed to quantify VPA.

The association between VPA and hepatotoxicity is well recognized.[35–40] Acute VPA intoxication also occurs as a consequence of intentional or accidental overdose.[11,13,41–44] Fatal overdose is rare and only a few suicidal cases are available in the published literature.[45–48]

In this study, we report a suicidal case involving VPA. A specific, fast and sensitive UPLC–MS/MS method was developed to detect, confirm and quantify VPA in the postmortem samples, providing a useful and robust technique to be used in routine forensic investigation.

2. Case report

A 45-year-old female, was found dead by her husband, lying in bed with five empty blisters-packs of *Diplexil*® next to her. She was a chronic alcoholic and epileptic who had been under psychiatric treatment, having repeatedly demonstrated intent to commit suicide.

At the autopsy, no signs of traumatic injuries were registered. The internal examination was unremarkable except for some tissue congestion and edema. Pancreas had no evidence of hemorrhage, fatty necrosis, fibrosis or calcifications. Weights of all organs were within normal ranges except the liver with an acute augmented volume. In addition, there were unidentified white fragments present in the stomach contents. Histological findings showed an exuberant congestion of the kidneys and lungs as well as hepatic steatosis.

Samples of blood, liver, kidney and stomach contents were taken for toxicological analysis.

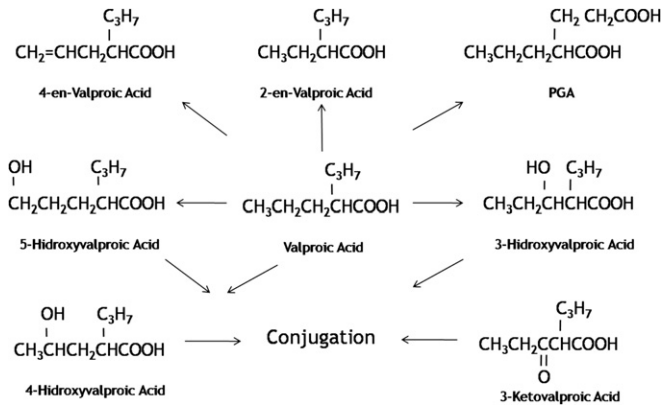

Fig. 1. Metabolic pathways for valproic acid.[1]

3. Materials and methods

3.1. Chemicals and reagents

Sodium valproate was supplied by Ciba–Geigy (New Jersey, USA) and hydrochlorothiazide (IS) by Sigma–Aldrich-Chemie GmbH (Steinheim, Germany). Ammonium acetate (analytical grade) was purchased from Riedel-deHaën (Seelze, Germany), and ortho-phosphoric acid analytical grade was from Merck (Darmstadt, Germany). Drug-free human blood was obtained from the Portuguese Blood Institute (Coimbra, Portugal) and was stored at −20 °C. Methanol analytical grade and LC–MS-grade water were purchased from Merck (Darmstadt, Germany), while acetonitrile LC–MS-grade was from Fisher Scientific (Leicestershire, UK). The mobile phase was filtered through a 0.20 μm filter (Schleicher & Schuell) and degassed in an ultrasonic bath for 15 min prior to use. Oasis HLB® SPE Cartridges (60 mg, 3 mL), used for sample preparation, were from Waters (Waters® Corporation, Milford, MA, USA), and were used on a solid-phase system Vac Elut EPS 24 from Varian (Harbor City, CA, USA).

Stock standard solutions of VPA and IS were prepared in methanol at concentrations of 1 mg/mL and stored at 4 °C. Working standard solutions were prepared by dilution of these stock solutions to appropriate concentrations. These standard solutions were stored at 4 °C.

3.2. Instrumentation

An ACQUITY Ultra Performance LC system (Waters® Corporation, Milford, MA, USA), composed of a binary solvent delivery manager, a thermostatted autosampler and column over compartment was used. Separation was performed on an Acquity UPLC® BEH C_{18} column, 2.1 × 50 mm, packed with 1.7 μm particles, which was maintained at 50 °C.

The mobile phase, consisting of acetonitrile (solvent A) and ammonium acetate buffer 10 mM (solvent B), was delivered at a flow rate of 0.50 mL/min. The gradient program was as follows: initial 98% B for the first 3.5 min, then gradient elution was performed by changing the mobile phase from 98% to 80% B between 3.5 and 4.3 min, and to 5% B between 4.3 and 5.0 min, after this time reversion of the mobile phase to 98% B. At the end of this sequence the column was equilibrated under initial conditions for 2.0 min. The autosampler temperature was set at 15 °C. The system was equipped with strong and weak wash solution reservoirs.

Detection was carried out using an Acquity™ TQD tandem-quadrupole MS equipped with a Z-spray electrospray ionization (ESI) source (Waters® Corporation, Milford, MA, USA) operating in negative mode. Argon was used as collision gas, and nitrogen was used as the nebulizing and desolvation gas.

The optimized MS settings employed for both VPA and IS were developed and maintained at the following: capillary voltage (3.0 kV), extractor voltage (2.0 V), RF lens voltage (0.1 V), source temperature (150 °C), desolvation temperature (350 °C), cone gas flow rate (0 L/h), desolvation gas flow rate (750 L/h), multiplier voltage (650 V) and collision gas flow (0.15 mL/min).

VPA and IS were detected in multiple reaction monitoring (MRM) mode using mass-to-charge (m/z) transitions of 143.1–143.1 and 296.1–205.0 respectively.

All aspects of system operation and data acquisition were controlled using Masslynx™ v4.1 software (Waters® Corporation,

Milford, MA, USA) with automated data processing using the TargetLynx™ Application Manager (Waters® Corporation, Milford, MA, USA). IntelliStart™ software (Waters® Corporation, Milford, MA, USA) was used to control the fluidics device to infuse solutions for tuning the MS.

3.3. Sample preparation

Control and calibration samples were prepared by spiking drug-free blood samples with standard solutions.

A 0.5 mL aliquot of blood sample was spiked with 50 μL of working solution (50 μg/mL) of IS and vortex-mixed. The samples were treated with 50 μL of 10% ortho-phosphoric acid (v/v) followed by addition of 2 mL water.[32] The mixture was vortex-mixed for 30 s following centrifugation at 2000×*g* for 5 min and loaded onto HLB cartridge, which had been conditioned with 2 mL methanol followed by 2 mL water. The washing of the cartridges was carried out with 5% (v/v) aqueous methanol (2 mL). Vacuum was applied on the solid-phase system for 10 min to dry the solvent. VPA and IS were eluted with 2 mL methanol. The elution was evaporated to dryness using a Turbo Vap LV evaporator (Caliper, Hopkinton, MA, USA) at 40 °C under nitrogen steam. The dried extract was then reconstituted with 200 μL of water and 10 μL was injected into the UPLC–MS/MS system in full loop mode. The kidney and liver postmortem samples were treated similarly to blood, although with some slight changes. Water (3 mL) was added to the samples (1 g) and then the mixture was sonicated for 2 min and centrifuged at 2000×*g* for 5 min. After that, total upper layer was used and treated as described before.

4. Results and discussion

The blood sample was initially subjected to a qualitative analysis. Screening was performed for basic, acidic, and neutral drugs and volatiles using standard methods. These included gas and liquid chromatography and enzyme immunoassays. Toxicological results showed high blood concentrations of VPA and therapeutic concentrations of tiapride, mirtazapine, oxazepam and nordiazepam. The blood alcohol analysis result (analyzed by a headspace GC/FID technique) was found positive (1.34 g/L).

This analytical method was developed and validated for assaying VPA in therapeutic concentration range for the analysis of routine samples. To achieve this aim, sample extraction procedure, liquid chromatography conditions and MS parameters were optimized.

Another solid-phase packing material, Oasis® MAX (500 mg, 6 mL) was tested for the present application. However, the Oasis HLB® cartridge was found to meet the criteria of cleaner injection extracts and higher recovery.

Prior to SPE we used the same sample treatment as described by Jain et al.[32] Addition of o-phosphoric acid helped in breaking the protein binding and contributed significantly in reducing matrix interactions.

Experiments were also performed in order to evaluate the influence of the diluents for the reconstitution set of the dried extracts on MS response. High recovery and good peak shapes were obtained using 100% water.

The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution for the VPA and IS. The ESI of VPA and IS produced the $[M - H]^-$ ions at 143.1 and 296.1, respectively, under negative ion mode. No marked fragment ions were observed in MS/MS spectra for VPA, therefore, quantitative analysis was performed by employing MRM using the non-reactive transition m/z 143.1 > 143.1 for VPA. The use of MRM, even when the same m/z value was employed for both precursor and product ions, allowed the background interference to be significantly reduced.[33]

Test for selectivity was carried out in 10 lots of drug-free human blood. The method employed for extraction gave good results in terms of selectivity and sensitivity for the analysis of VPA and IS in blood (Fig. 2). All chromatograms were free of background interference.

The calibration curves for VPA in the blood samples were linear, ranging from 0.5 to 100 μg/mL ($y = 1.3243x - 0.4536$ with $r^2 = 0.999$, for $n = 10$). The limit of quantification (LOQ) was defined in this study as the lowest calibrator with an acceptable relative uncertainty (LOQ = 0.5 μg/mL). The limit of detection (LOD) was evaluated by decreasing concentrations of VPA until the response of quantitative ion was equal to three times the response of the blank extract (LOD = 0.1 μg/mL). Analytical recovery was tested at

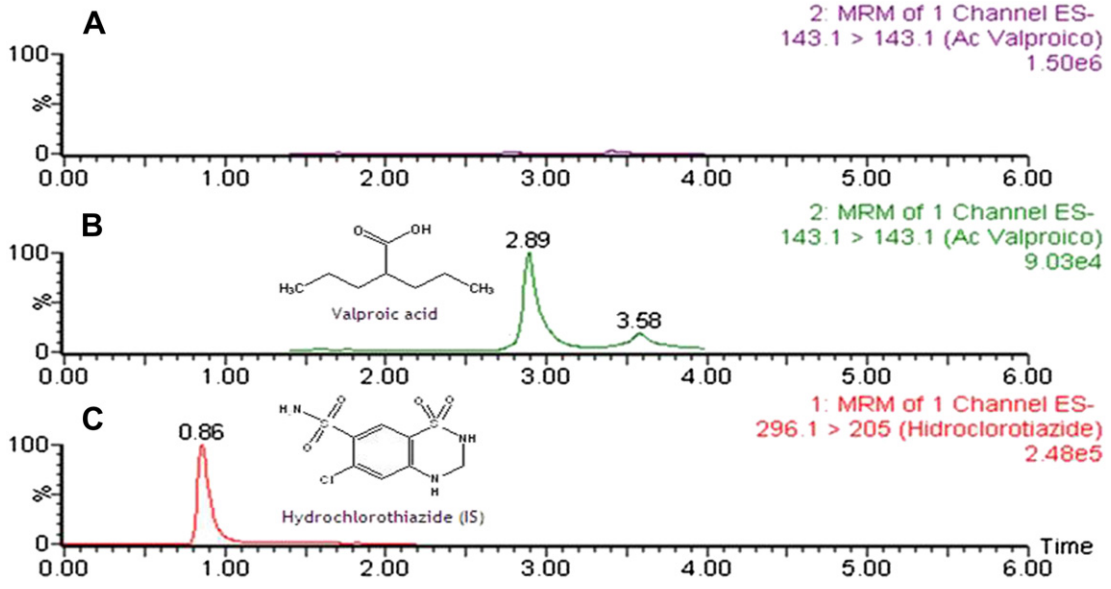

Fig. 2. MRM mass spectra of a blank blood sample (A), 0.5 μg/mL (LOQ) of VPA (B) and IS (C).

Table 1
Validation data of precision, accuracy and recovery of valproic acid in blood.

Conc. added (μg/mL)	Intra-day precision ($n = 3$)			Inter-day precision ($n = 5$)			Recovery (%) ($n = 3$) (mean ± SD)
	Conc. found (mean ± SD; μg/mL)	Precision (%)	Accuracy (%)	Conc. found (mean ± SD; μg/mL)	Precision (%)	Accuracy (%)	
5	4.5 ± 0.32	7.1	90.3	4.7 ± 0.30	6.2	97.2	94.4 ± 11.5
10	10.2 ± 0.57	5.6	102.2	9.8 ± 0.26	2.6	92.2	96.1 ± 3.5
50	48.9 ± 3.29	6.7	97.8	48.5 ± 1.5	3.1	96.4	90.3 ± 2.6

concentration levels 5 μg/mL, 10 μg/mL and 50 μg/mL and the mean recovery was 95% (see Table 1). Very high and reproducible recoveries for VPA were obtained with this SPE procedure. For intra-day and inter-day precision determinations, three replicate analyses were performed on each of the three concentrations studied. The method proved to be accurate for VPA, both in terms of intra-day and inter-day analysis, with coefficients of variation (CV) of less than 10%. Relevant validation data for recovery, accuracy and precision are presented in Table 1.

Since the procedure proved to be sensitive, selective and reproducible, the method developed was applied to the fatal case presented. VPA was detected in all the specimens analyzed. Toxicological results revealed the following VPA concentrations in the postmortem samples: blood 556 μg/mL; kidney 69 μg/mg; liver 104 μg/mg; stomach contents 22.4 mg (in 50 mL). Therapeutic concentrations of other psychiatric drugs such as tiapride (0.53 μg/mL), mirtazapine (0.08 μg/mL), nordiazepam (0.07 μg/mL) and oxazepam (0.39 μg/mL) were found in this case.

Acute VPA intoxication occurs as a consequence of intentional or accidental overdose.[11,13,41–44] Fatal overdose is rare and only a few suicidal cases are available in the published literature.[45–49]

Comparison of our quantitative results for VPA to those found in literature shows that the measured values are in the same range. The ratios of VPA concentrations in the tissues to those of blood are 0.12 for the kidney and 0.19 for the liver. This is consistent with the relative low apparent volume of distribution of VPA, 0.1 to 0.5 L/kg. VPA was present in stomach contents, indicating acute VPA intoxication.

Tiapride, mirtazapine, nordiazepam and oxazepam were also present but there is a lack of published evidence of drug–drug interaction for these drugs with VPA. Although VPA usually is well tolerated, several authors[6,8,50] refer that its use in combination with other psychotropic compounds might bear an elevated risk of adverse reactions.

After excluding death due to natural or traumatic causes, a direct toxic effect by VPA was considered. Taking into account the autopsy, histopathology and toxicological findings, along with the circumstantial evidence, the cause of death was attributed to suicide by intoxication with VPA in association with other CNS depressants.

This method offers significant advantages over those previously reported, in terms of improved sensitivity and selectivity, faster run time and rapid extraction (without chemical derivatization). This method provides a useful and robust technique to be used in routine forensic investigation.

Conflict of interest

Authors have no financial or personal conflict of interest regarding this manuscript.

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Ethical approval

None.

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